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Supplementary Tables.

Supplementary Table 1. Fungal X325 enzymes that have been recombinantly produced in *Pichia pastoris*.

Name	Fungi	Protein ID
<i>LbX325</i>	<i>Laccaria bicolor</i>	XP_001874295.1
<i>LaX325</i>	<i>Laetisaria arvalis</i>	MK088083
<i>PaX325</i>	<i>Podospora anserina</i>	XP_001907559.1
<i>YIX325</i>	<i>Yarrowia lipolytica</i>	XP_505821.1

Supplementary Table 2: Crystallographic data collection and refinement statistics.

	<i>LaX325 P2₁2₁2₁</i>	<i>LaX325 P4₃2₁2</i>	<i>LaX325 P2₁</i>
Data collection			
Space group	<i>P2₁2₁2₁</i>	<i>P4₃2₁2</i>	<i>P2₁</i>
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	84.60 , 84.64 , 127.32	74.92, 74.92, 64.39	32.94, 67.59, 68.90
α , β , γ (°)	90.000, 90.000, 90.000	90.000, 90.000, 90.000	90.000, 97.939, 90.000
Mol/ASU	4	1	2
Resolution (Å)	100 - 2.08 (2.13 – 2.08)*	60 - 2.10 (2.15 – 2.10)	68.25 -1.82 (1.87 – 1.82)
<i>R</i> _{meas}	0.152 (1.398)	0.087 (1.703)	0.097 (0.911)
<i>I</i> / σ <i>I</i>	12.93 (1.07)	12.25 (1.49)	11.79 (2.12)
Completeness (%)	99.6 (98.5)	99.9 (99.9)	95.1 (68.9)
Redundancy [§]	14.0 (14.3)	7.2 (7.4)	7.4 (6.3)
No. of reflections	775853 (55401)	80479 (6008)	188926 (8457)
No unique of reflections	57268 (4005)	11173 (807)	25629 (1351)
CC _{1/2} (%)	99.8 (74.4)	99.9 (80.3)	99.7 (81.6)
Refinement			
Resolution (Å)	42.35 - 2.08 (2.13 – 2.08)	48.88 - 2.10 (2.15 - 2.10)	68.24 -1.82 (1.86 – 1.82)
No. reflections	52714 (3752)	10600 (575)	24384 (1262)
<i>R</i> _{work} / <i>R</i> _{free} (%)	24.4 / 27.1 (45.1 / 49.0)	19.35/ 25.6 (34.3 / 34.1)	16.7 / 20.8 (33.9 / 32.8)
No. atoms			
Protein [§]	A: 1257, B : 1215, C : 1276, D : 1184	A: 1182	A : 1241, B : 1209
Solvent [#]	614	55	192
<i>B</i> -factors (Å ²)			
Protein	A: 39.1, B: 43.5 C: 43.8, D: 55.5	A: 57.6	A : 38.5 B : 40.8
Solvent	50.4	58.0	46.7
R.m.s. deviations			
PDB code	6IBI	6IBJ	6IBH

One of crystal was used for each structure.

*Values in parentheses are for highest-resolution shell.

§ Friedel pairs are treated as symmetry-equivalent reflections

§ Glycosylation (N-acetylglucosamine units) and the active site copper are included in “Protein”

PEG, imidazole and water molecules are included under “Solvent”

Supplementary Table 3. Cu-ligand distances and geometry of coordination.

Protein	Spacegroup	mol	Equatorial ligand positions (Å)				axial positions (Å)		Cu-ligand angles (°) [□]			PDB
Macromolecules			Cu-NH ₂	Cu-Nδ	Cu- Nε/(Nδ)	Cu-O	Cu-O [§]	Cu-H ₂ O _{ax}	Θ1	Θ2	Θ3	
<i>LaX325</i>	<i>P2₁2₁2₁</i>	A	2.04	2.03	2.06	2.00	(2.88)	2.51	92.4	88.5	169.6	6IBI
<i>LaX325</i>	<i>P2₁2₁2₁</i>	B	2.03	2.06	2.00	2.02	(2.99)	N/A	91.3	90.4	176.1	6IBI
<i>LaX325</i>	<i>P2₁2₁2₁</i>	C	2.08	2.02	2.03	2.07	(2.97)	2.35	90.4	88.3	178.7	6IBI
<i>LaX325</i>	<i>P2₁2₁2₁</i>	D	2.02	2.01	2.05	2.02	(3.07)	N/A	92.9	88.8	178.3	6IBI
<i>LaX325</i>	<i>P4₃2₁2</i>	A	2.02	2.04	2.03	1.98	(3.00)	N/A	91.7	86.0	175.4	6IBJ
<i>LaX325</i>	<i>P2₁</i>	A	2.04	2.03	2.03	2.09	(2.86)	2.65	94.5	84.7	177.8	6IBH
<i>LaX325</i>	<i>P2₁</i>	B	2.05	2.05	2.04	2.08	(2.82)	2.28	94.6	84.5	176.5	6IBH
Mean and standard deviations			2.04 ±0.02	2.03 ±0.02	2.03 ±0.02	2.04 ±0.04	2.94 ±0.09	2.45 ±0.17	92.5 ±1.6	87.1 ±2.4	176.1 ±3.1	
<i>TdAA15</i> [§]	<i>P22₁2₁</i>	A	2.16	2.00	1.99	N/A	2.56	N/A	96.0	94.2	169.2	5MSZ
<i>CopC</i> [*]	<i>P2₁2₁2₁</i>	A	2.03	2.06	1.97*	2.30*	N/A	2.37	91.5	(79.1)*	(170.3)*	5ICU
<i>NcAA9F</i> [#]	<i>P2₁2₁2₁</i>	B	2.12	1.96	1.98	2.16 [#]	2.60	2.68 [#]	92.3	94.3	172.2	4QI8

□ Θ1: NH₂-Cu-Nδ; Θ2: NH₂-Cu-Nε; Θ3 Nδ-Cu-Nε.

§ Tyr occupy this position in *TdAA15* and *NcAA9F*. *LaX325* does not have an equivalent Tyr, but the Asp122 side chain oxygen not providing equatorial coordination is found at a 2.94±0.09 Å distance to the Cu (distorted compared to octahedral coordination).

* in *CopC*, these equatorial ligands are swapped compared to the other Cu sites, and the Cu is coordinated by Nδ rather than Nε (Consequently, for *CopC* the angles are defined as Θ2: NH₂-Cu-O and Θ3: Nδ-Cu-O).

Asp33 from a symmetry-related molecule occupy these ligand positions with distorted geometry

Supplementary Table 4: EPR spin Hamiltonian parameters from simulations of cw X band spectra. *LaX325* was analysed in 50 mM Na acetate buffer pH 5.2.

<i>LaX325</i>		
g values	g_x	2.040
	g_y	2.076
	g_z	2.260
A_{Cu} (MHz)	$ A_x $	42
	$ A_y $	64
	$ A_z $	543
SHF A_N (principal values) (MHz)		28, 32, 36
A_{cu} strains (MHz)		46, 80, 80
Line widths		0.5, 0.4
Frequency (GHz)		9.315043

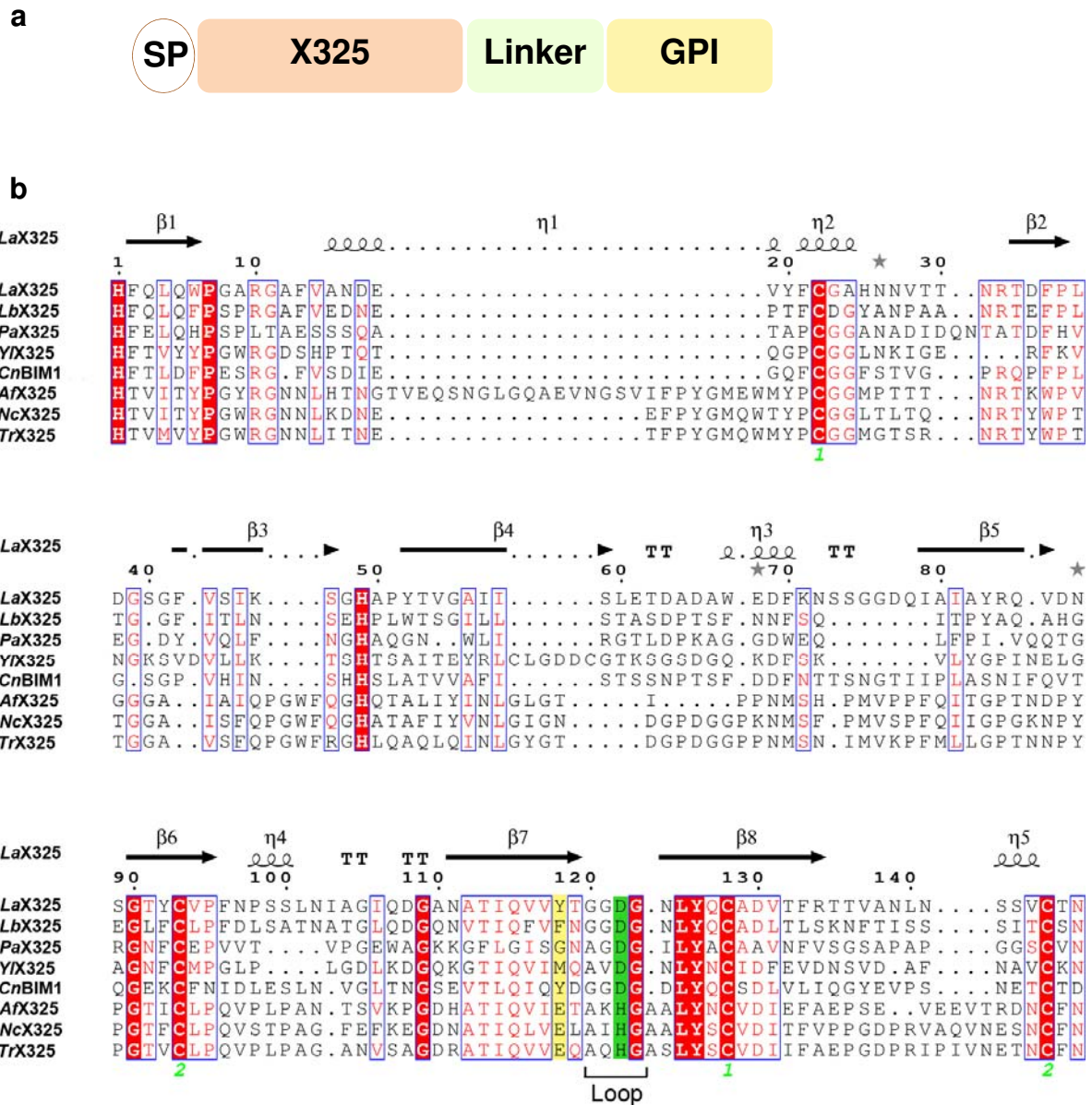
Supplementary Table 5. X325 encoding genes upregulated in some symbiotic fungi. Cenge = *Cenococcum geophilum*, Tubma = *Tuber magnatum*, Oidma = *Oidiodendron maius*, Melbi = *Meliniomyces bicolor*, Rhier = *Rhizoscyphus ericaceae*. FLM: free-living mycelium; ECM: ectomycorrhizal; FC: fold change; FDR: false discovery rate. Each expression represents the mean of triplicate values. *Students't-test: values were calculated by comparing relative quantification measured in ECM with that in FLM.

Ectomycorrhizae				
X325 encoding genes	FLM mean expression	ECM mean expression	FC ECM vs FLM	FDR <i>p</i>-value*
Cenge 644941	185	412	2.2	6.27E-06
Tubma 360409	5	474	91.5	3.83E-01
<i>LbX325</i>	56	354	11.3	1.20E-09
Ericoid mycorrhizae				
Oidma 153540	64	250	3.9	3.44E-21
Melbi 531740	29	108	3.8	2.72E-04
Rhier 571157	26	440	16.9	5.15E-07
Rhier 645372	1	22	16.1	1.38E-06

Supplementary Table 6. Statistical parameters of Kolmogorov-Smirnov tests performed on the immunogold labelling data (Supplementary Figure 8). FLM, free living mycelia; ECM, ectomycorrhiza.

	FLM		ECM	
	WGA	<i>LbX325</i>	WGA	<i>LbX325</i>
min	-0.598	-0.752	-1.957	-1.207
Q1	-0.218	-0.002	-0.183	-0.057
med	-0.035	0.311	0.016	0.188
Q3	0.214	0.536	0.267	0.494
max	1.457	1.371	2.509	2.013
n	161	161	161	161

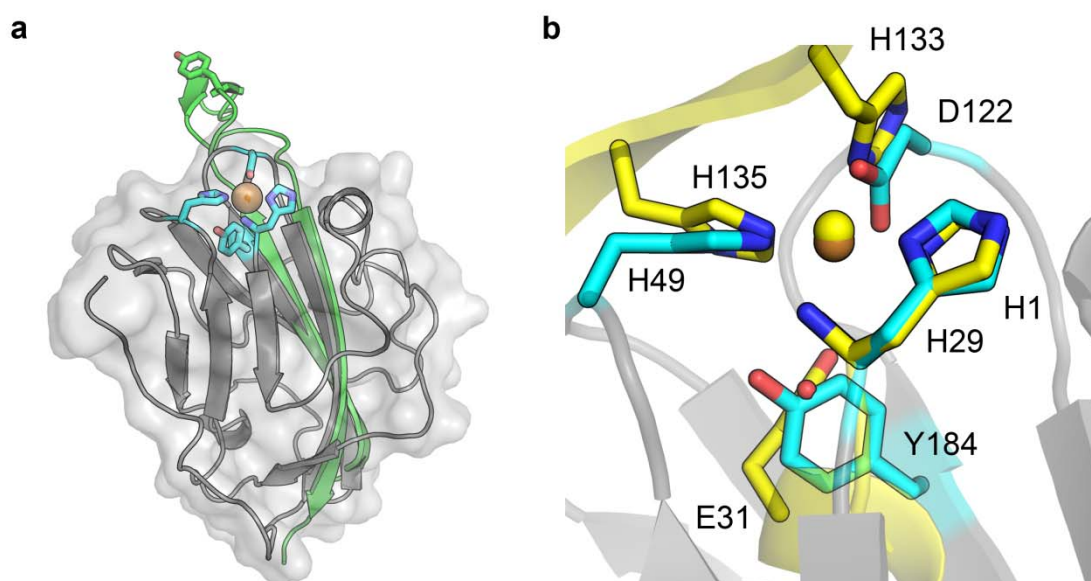
Supplementary Figures



Supplementary Figure 1. Modularity (a) and structure-based sequence alignment (b) of family X325.

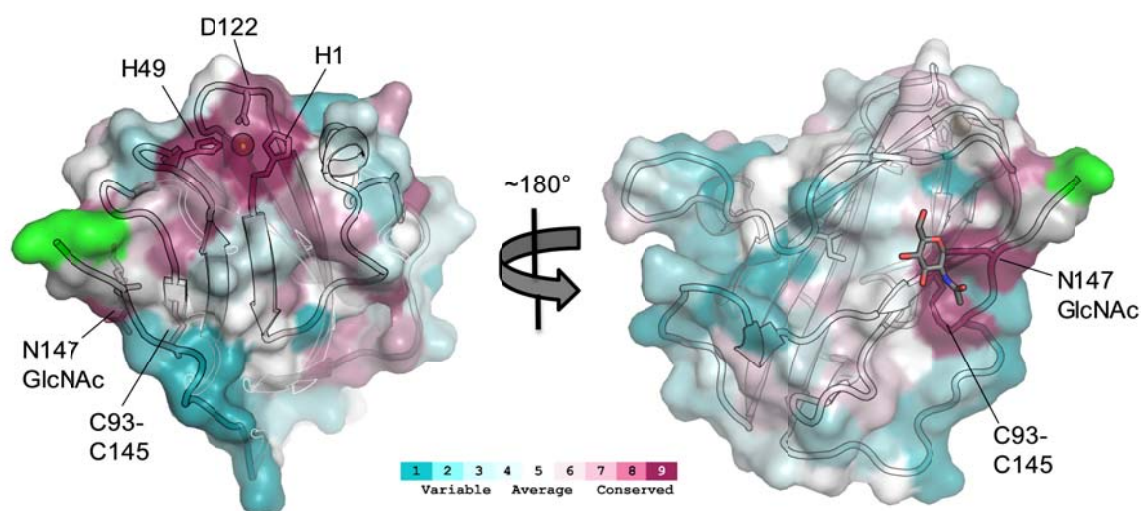
(a) In the N-terminal region, the signal peptide (SP) is followed by the X325 catalytic module. A linker separates the catalytic module and the C-terminal glycosylphosphatidylinositol (GPI) anchor. (b) The catalytic module of *LaX325* is compared with some others X325 members. The helices and strands are represented as *helices* and *arrows*, respectively, and turns are marked with *TT*. This sequence alignment was created using the sequences coming from the following organisms: *LaX325* (*Laetisaria arvalis*, MK088083, residues 1–147), *LbX325* (*Laccaria bicolor*, GenBank ID XM_001874260.1, residues 1–139), *PaX325* (*Podospira anserina*, GenBank ID XM_001907524.1, residues 1–135), *YIX325* (*Yarrowia lipolytica*, GenBank ID XM_505821.1, residues 1–140), *CnBIM1* (*Cryptococcus neoformans* X325,

GenBank ID XM_569628.2, residues 1–144), AfX325 (*Aspergillus fumigatus*, GenBank ID KEY79179.1, residues 1–173), NcX325 (*Neurospora crassa*, GenBank ID XM_011394541.1, residues 1–163), and TrX325 (*Trichoderma reesei*, GenBank ID XM_006963964.1, residues 1–163). *Dark shaded boxes* enclose invariant positions, and *light shaded boxes* highlight positions with similar residues. *Green numbers* highlight the cysteines involved in disulfide bonds. The *yellow* highlights the position of the Tyr118 in LaX325 structure, which is not conserved across the X325 family. The *green* highlights the position of the Asp122 in LaX325 structure. The figure was created with ESPript (<http://esprict.ibcp.fr>).



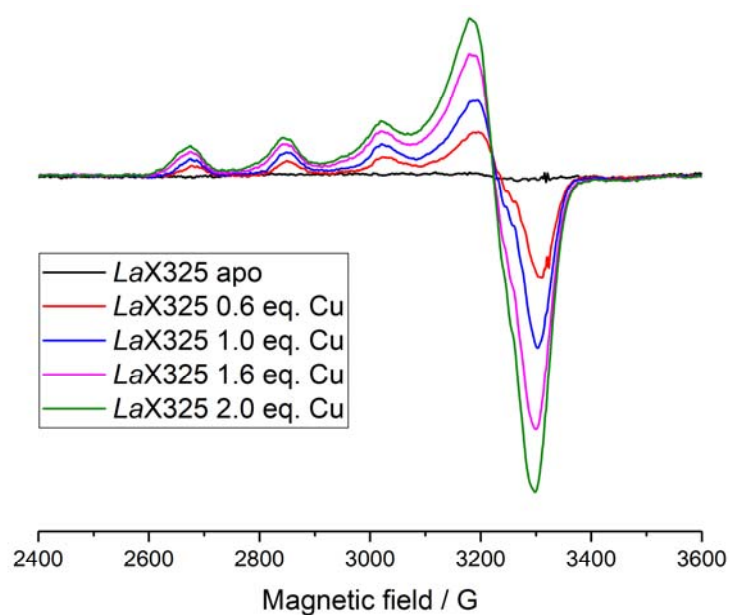
Supplementary Figure 2. Structure comparison of LaX325 to TdAA15A and the Cu_B copper center of particulate methane monooxygenases (pMMOs).

(a) Superimposition of TdAA15 (*green*) onto LaX325 (*grey*). Of the LaX325 active site residues (*cyan*), Asp122 is found on a short loop equivalent to the AA15 extended protruding loop bearing a conserved Tyr (*green*). **(b)** Superimposition of copper binding sites of LaX325 (*cyan and copper*) and the Cu_B center of pMMO from *Methylocystis sp.* strain M (*yellow*, Chain I of PDB entry 3RFR). A subgroup of X325 displays a His instead of Asp (Asp122 in LaX325) in one of the equatorial Cu-coordinating ligand positions, and in addition a Glu instead of the Tyr near the active site copper (Tyr184 in LaX325), which bears some resemblance to pMMOs.



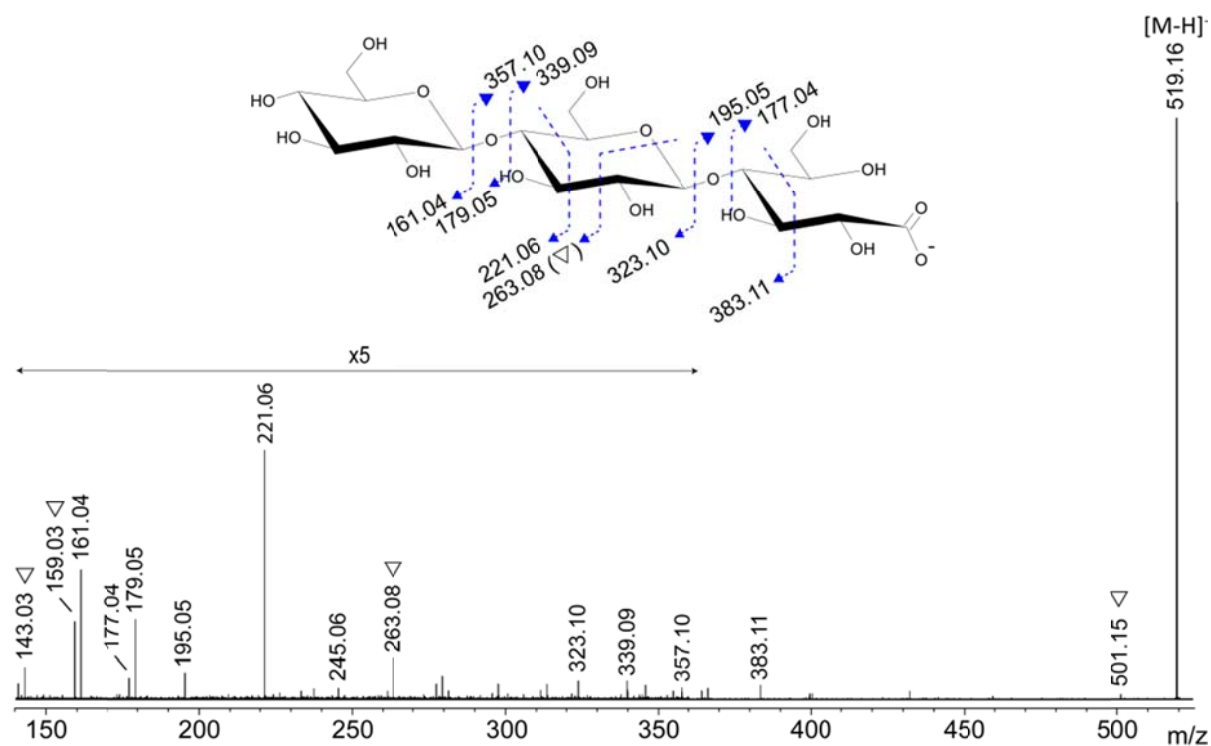
Supplementary Figure 3. Sequence conservation mapped onto the *LaX325* structure.

The *LaX325* structure (PDB 6IBJ) is shown with surface color according to sequence conservation within the X325 protein family using the 123 sequences used to build the phylogenetic tree (**Figure 1**). Conserved regions are shown in magenta and variable regions in cyan. The Cu-coordinating residues His1, His49 and Asp122 are highly conserved (left). A second conserved patch is found near the C-terminus (green) formed by a disulfide bond (Cys93-Cys145) and Asn145 (involved in N-linked glycosylation). The figure was prepared using ConSurf (<http://consurf.tau.ac.il/2016/credits.php>).

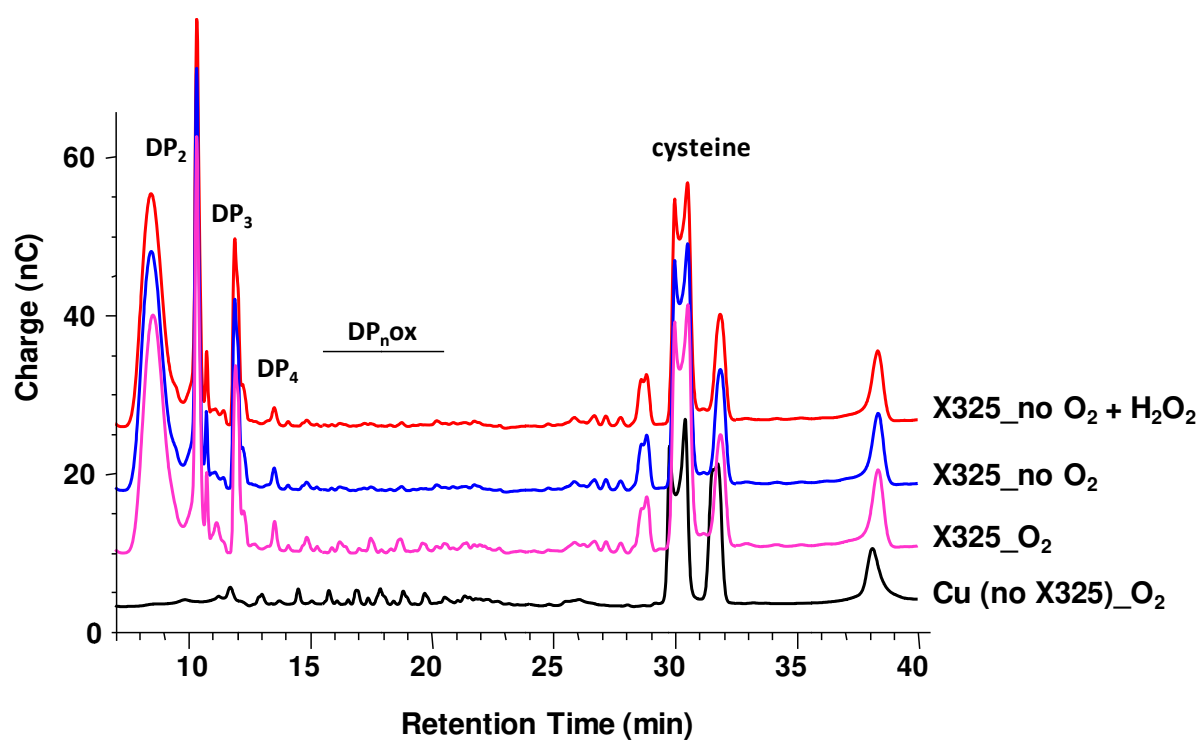


Supplementary Figure 4. Continuous wave X-band EPR Cu titration spectra (~9.3 GHz, 165 K) of *LaX325*. For clarity, only a selection of spectra is presented; full data are available through the Research Data York (DOI: [10.15124/a034974e-2782-415e-8b02-2b6e4098760e](https://doi.org/10.15124/a034974e-2782-415e-8b02-2b6e4098760e))

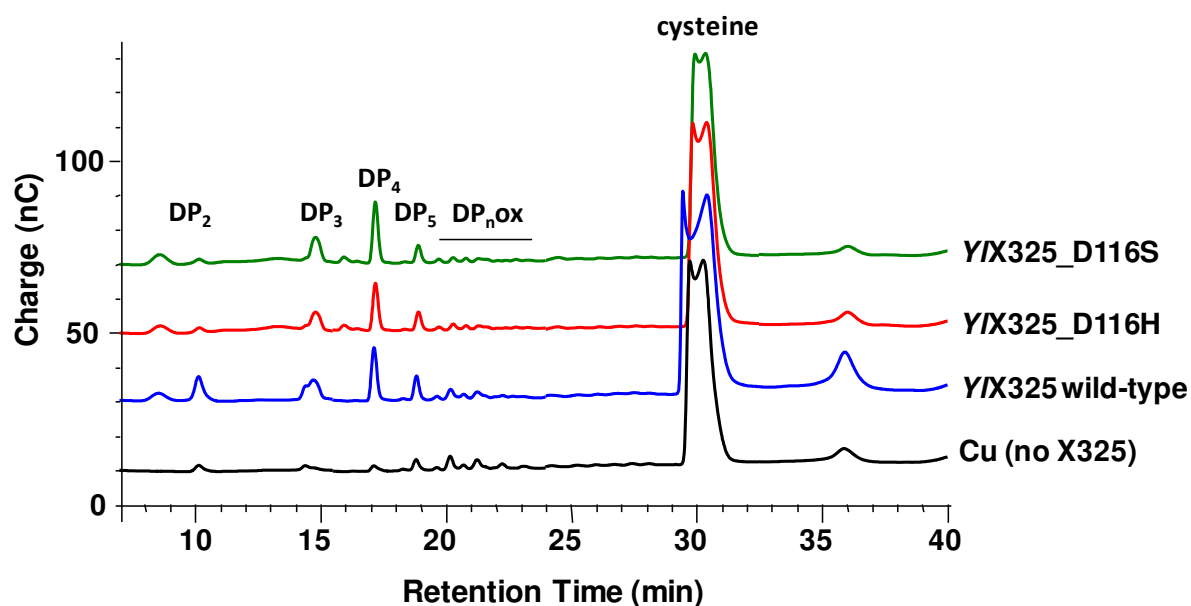
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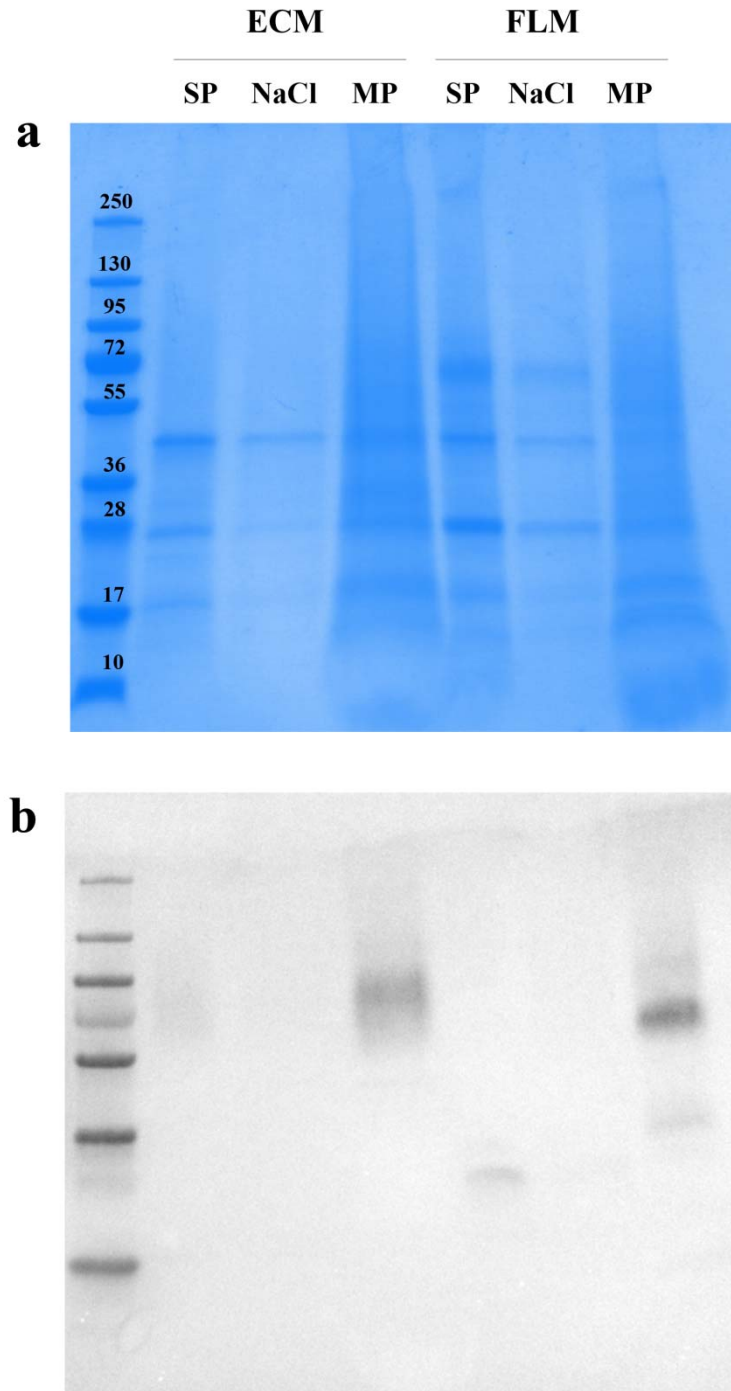
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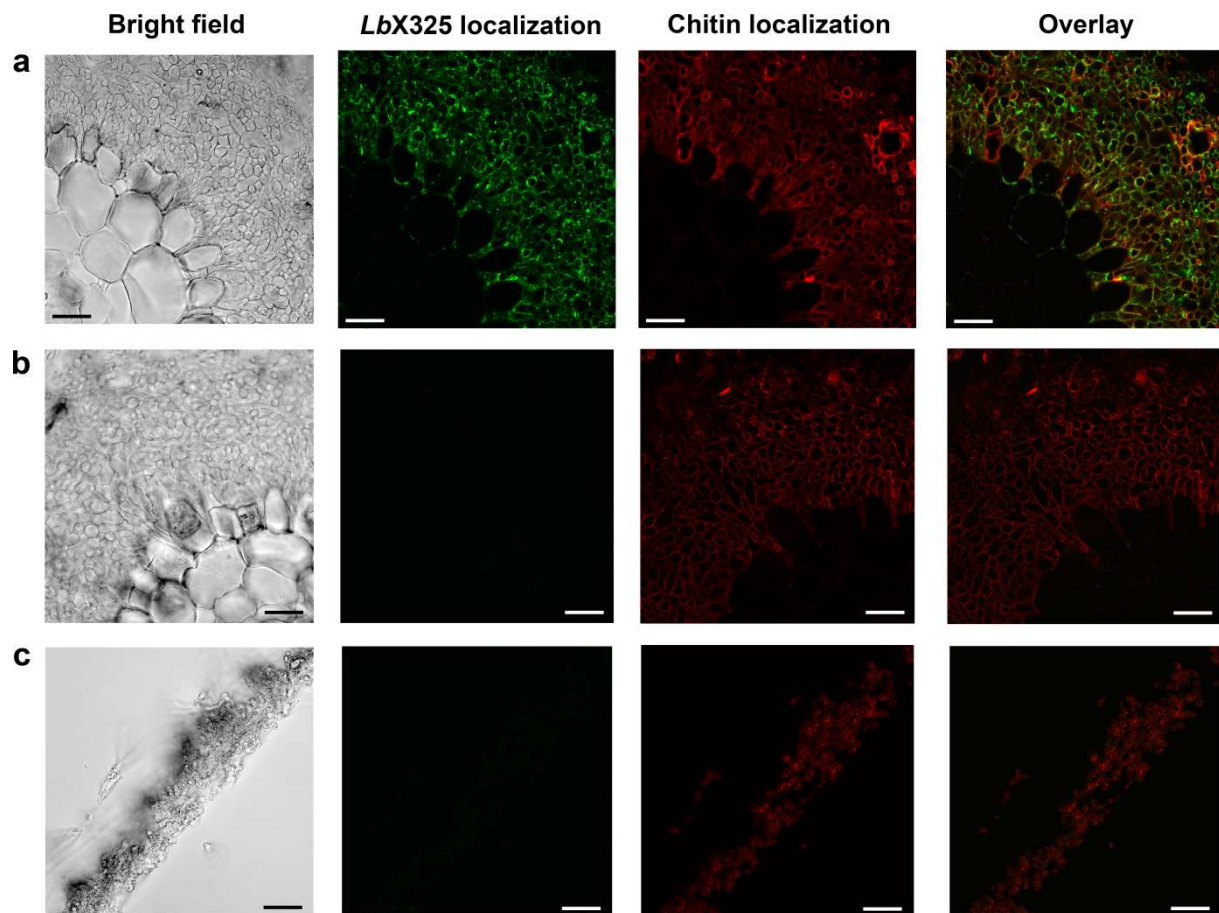
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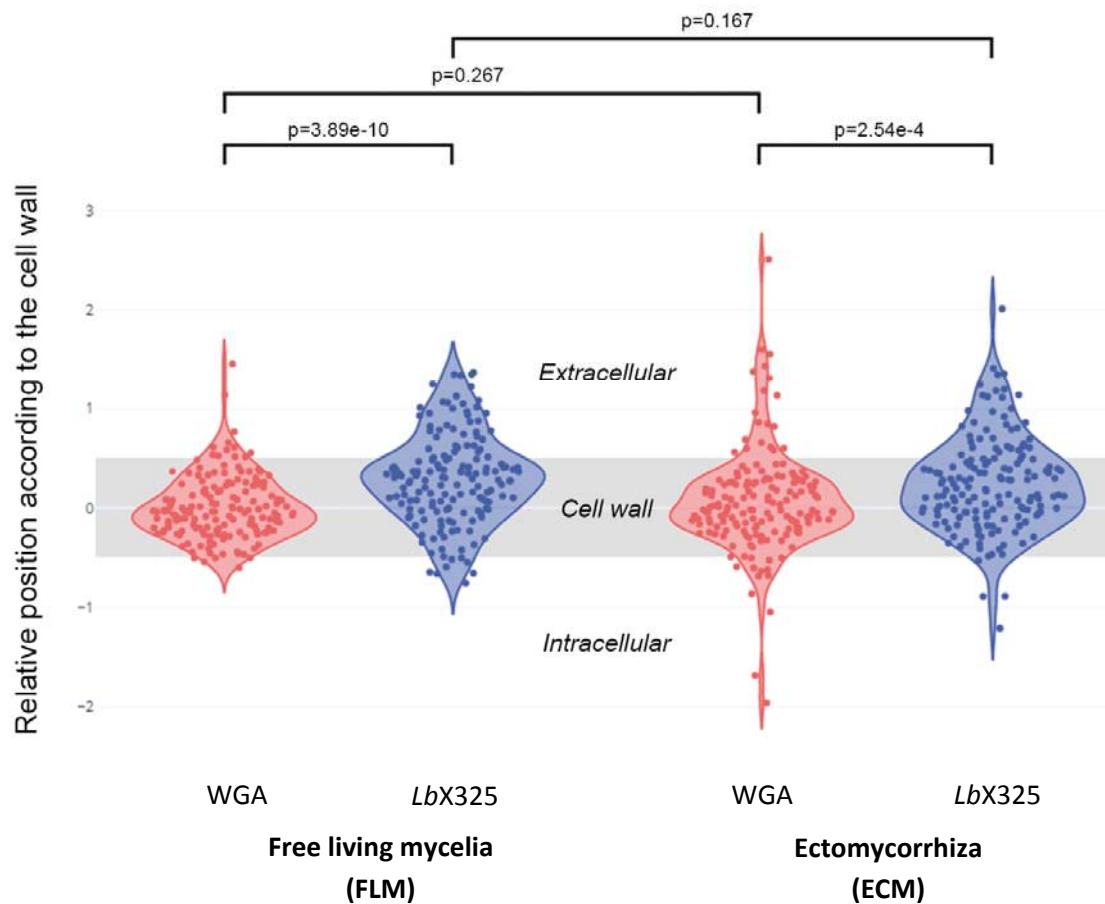
Supplementary Figure 5. Detection of soluble products generated by the action of X325 proteins on cellulose. (a) MS/MS identification of the G3 oxidized species detected at 519.16 m/z generated from Avicel by *LaX325*. The fragmentation pattern corresponds to a C1 oxidized species with an aldonic acid at the reducing end. The structure represents a C1 oxidized cellotriose deduced from the spectrum. ∇ : water losses. (b) Polysaccharides cleavage assays under anaerobic conditions. Reactions were carried out as described in material and methods using 5 μ M X325 proteins or CuSO_4 with and without O_2 . Addition of 50 μ M of H_2O_2 was also attempted under anaerobic conditions. Chromatograms are representative of triplicate independent experiments. Peaks were assigned according to standards. (c) Polysaccharides cleavage assays following mutagenesis of the Asp116 residue of *Y/X325* (equivalent to Asp122 in *LaX325*) to either Ser (D116S) or His (D116H). Reactions were carried out as described in material and methods using 1 μ M X325 wild type or mutant proteins or CuSO_4 . Chromatograms are representative of triplicate independent experiments. Peaks were assigned according to standards.



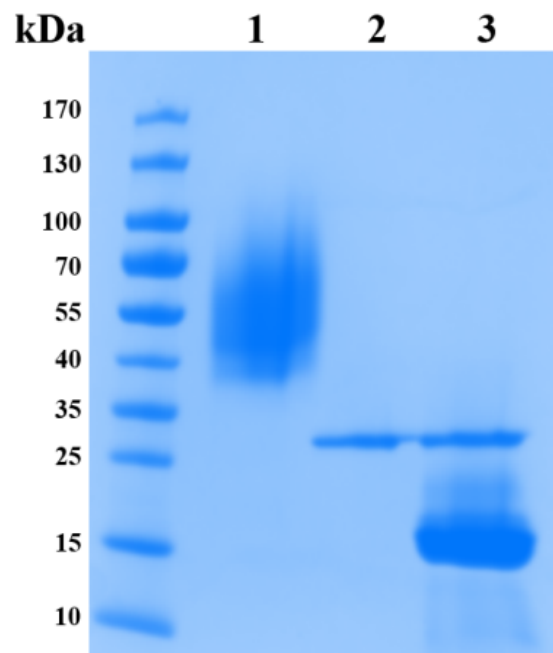
Supplementary Figure 6. Protein electrophoresis of *L. bicolor* S238N crude extracts (a) and the corresponding western immunoblotting using antibodies raised against the purified recombinant *LbX325* (b). Data were acquired from a single experiment. ECM = ectomycorrhizal roots, FLM = free living mycelia, SP = soluble protein fraction, NaCl fraction = proteins not covalently bound, MP = proteins attached to the membrane. For both gels, 20 μ l of each crude extract fractions were loaded. Protein Ladder Range is indicated in kDa. See materials and methods.



Supplementary Figure 7. Immunolocalization of *LbX325* in *Populus-Laccaria bicolor* ectomycorrhiza. All images were obtained by using indirect immunofluorescence confocal laser microscopy, except the bright field images. **(a)** Transverse sections of 3-weeks-old ectomycorrhiza stained for *LbX325* with anti-*LbX325* immune serum (green) and for chitin with wheat germ agglutinin (WGA) (red). **(b)** Binding of the anti-*LbX325* antibodies was blocked by incubating the sections with recombinant *LbX325* protein, confirming the specificity of the immune serum. **(c)** Sections of *L. bicolor* free-living mycelium stained for *LbX325* with anti-*LbX325* immune serum (green) and for chitin with WGA (red). Bars = 20 μm. Selected images are representative of five different sections.



Supplementary Figure 8. Quantitative approach of immunogold labelling on transverse sections of 3-weeks-old free living mycelia (FLM, left) and ectomycorrhiza (ECM, right) indicates a preferential localization of *LbX325* within and beyond the outer layers of the cell wall. The Wheat Germ Agglutinin (WGA) staining is used as a control for the fungal cell wall position (chitin). As expected, the relative position of the WGA staining coincide with the cell wall position defined by morphological analysis (grey area, after normalization, between positions -0.5 and +0.5, see material and methods). The WGA staining shows no significant difference in its relative position according to the cell wall center between the FLM and ECM conditions. In both FLM and ECM conditions, the *LbX325* staining shows a significant shift towards the outer layers of the cell wall. The statistical analysis was done carrying out Kolmogorov-Smirnov tests ($n=161$ beads for each staining within each condition). The test was two-sided. Statistical parameters are listed in **Supplementary Table 6**.



Supplementary Figure 9. SDS-PAGE analysis of *LaX325* control samples (lane 1), control endoH (lane 2) and endoH-deglycosylated *LaX325* sample (lanes 3) as described in materials and methods. 20 μ g of *LaX325* protein was loaded onto the gel. Data were acquired from a single experiment.